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Abstract: Sézary syndrome is a primary cutaneous T-cell lymphoma characterized by pruritic erythroderma, peripheral lymphadenopathy and the presence of malignant T cells in the blood. Unequivocal detection of malignant cells in patients with Sézary syndrome is of important diagnostic, prognostic and therapeutic value. However, no single Sézary syndrome specific cell surface marker has been identified. In a cohort of patients with Sézary syndrome, CD164 expression on total CD4+ lymphocytes was significantly upregulated compared with healthy controls. CD164 expression was in most cases limited to CD4+CD26- malignant T lymphocytes, unequivocally identified using flow-cytometry by the expression of a specific V clone for each patient. Increased expression of CD164 may be a promising diagnostic parameter and a potential target for a CD164-linked therapeutic approach in Sézary syndrome.

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INVESTIGATIVE REPORT

Expression of CD164 on Malignant T Cells in Sézary Syndrome

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Sézary syndrome is a primary cutaneous T-cell lymphoma characterized by pruritic erythroderma, peripheral lymphadenopathy and the presence of malignant T cells in the blood. Unequivocal detection of malignant cells in patients with Sézary syndrome is of important diagnostic, prognostic and therapeutic value. However, no single Sézary syndrome specific cell surface marker has been identified. In a cohort of patients with Sézary syndrome, CD164 expression on total CD4⁺ lymphocytes was significantly upregulated compared with healthy controls. CD164 expression was in most cases limited to CD4⁺CD26⁺ malignant T lymphocytes, unequivocally identified using flow-cytometry by the expression of a specific V β clone for each patient. Increased expression of CD164 may be a promising diagnostic parameter and a potential target for a CD164-linked therapeutic approach in Sézary syndrome. **Key words:** *Sézary syndrome; marker; CD164.*

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Cutaneous T-cell lymphomas (CTCL) are the second most common group of extranodal non-Hodgkin lymphomas, representing approximately 75% of all primary cutaneous lymphomas (1). Of all CTCL, mycosis fungoides and Sézary syndrome (SS) account for roughly 53% (2). SS, characterized by pruritic erythroderma, peripheral lymphadenopathy, and the presence of malignant T cells in the blood, has a poor prognosis, with a mean overall survival of approximately 5 years (3–5).

Easy and precise detection of malignant cells in the blood of patients with SS is of important diagnostic, prognostic and therapeutic value and is essential for disease monitoring under treatment (6–8). Currently, the loss of cell surface markers, such as CD26, CD27 and CD7, on malignant T lymphocytes remains one of the most consistent features of CTCL and is routinely used in the diagnostic workup (9), although their specificity, and especially sensitivity, must be interpreted with cau-

tion (10). However, a ubiquitously expressed, single clear-cut diagnostic marker allowing for the diagnosis of CTCL is yet to be discovered (11). An extensive gene expression microarray analysis, performed recently by Wysocka et al. (12) found that CD164 is upregulated in CD4⁺ T cells of patients with SS. The aim of the present study was to confirm CD164 as a potential marker for malignant T cells in the blood of patients with SS.

METHODS

Patients from the Department of Dermatology of the University Hospital of Zurich, Switzerland, with previously diagnosed SS and with unequivocally identifiable V β clonal T-cell population ($n=8$) were included in the study. Healthy individuals and patients with inflammatory skin disease (psoriasis) served as a control. The diagnosis of SS was established in all patients according to the World Health Organization–European Organization for Research and Treatment of Cancer criteria for SS (1).

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the University of Zurich (KEK-ZH-Nr. 2015-0209). Peripheral blood from the patients with SS and psoriasis was obtained from the University of Zürich Biobank, funded by the University of Zurich University Research Priority Program (URPP) in translational cancer biology. All patients agreed to the use of the blood, including the generation of cell cultures and other materials according to the Biobank project (EK No. 647). Blood from healthy individuals was obtained as discarded tissue from the blood bank of the University Hospital Zurich. All patients with CTCL were seen at the Cutaneous Lymphoma Clinic at the Department of Dermatology of the University Hospital of Zurich. Patients' characteristics and malignant T-cell burden are shown in Table I.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll centrifugation. Analysis of T cells was performed using monoclonal antibodies against human CD3 (clone BW264/56, label PerCP; Miltenyi Biotec #130-096-910), CD4 (Clone VIT4; label APC-Vio770; Miltenyi Biotec #130-098-153), CD26 (Clone BA5b, label PE-Cy7, #302714, BioLegend) and CD164 (clone N6B6; label FITC; BD Biosciences #551297). Isotype-matched negative control antibodies were used to set the gates for positive staining. V β clonal T-cell population was assessed by flow cytometry using the IOTest Beta Mark TCR V β Repertoire kit (Beckman Coulter) as previously described (8). Annexin V staining (label APC; BD Pharmingen #550474), paired with 7-AAD (label PerCP-Cy5; single reagent from the FITC BrdU Flow Kit; BD Pharmingen #559619) was used to

Table I. Patients' clinical characteristics at time of analysis

Pat. No.	TCR clone	Clonal cells %	Age, years/ Sex	CD4/CD8 ratio	CD4 ⁺ CD7 ⁻ %	CD4 ⁺ CD26 ⁻ %	Total lymphocyte count	Treatment	Disease duration, years
1	Vβ22	50	62/M	11.5	7	91	1,224	ECP, TS, UVB, B	1
2	Vβ13.2	24.81	61/M	46.9	14	94	18,776	ECP, IFN-α2a, PUVA	5
3	Vβ1	75.99	90/F	19.7	72	63	8,576	ECP, B, TS	11
4	Vβ14	91.5	70/F	72.7	86	97	5,522	ECP, MTX	6
5	Vβ7.1	69.8	67/M	21.2	37	49	1,417	PUVA	4
6	Vβ8	33.77	64/M	18.1	89	100	625	P, SST	6
7	Vβ1	85.44	72/F	13.6	10	99	5,996	ECP	2
8	Vβ22	90.25	74/M	28.7	91	99	1,551	ECP, IFN-γ	3

TCR: T-cell receptor; IFN: interferon; UVB: ultraviolet B; ECP: extracorporeal photochemotherapy; B: bexarotene; TS: topical steroids; MTX: methotrexate; P: pralatraxate; PUVA: psoralen + UVA treatment; SST: stem cell transplantation. Reference range: 850–2,500 lymphocytes/μl blood.

identify dimethyl-sulphoxide (DMSO)-induced apoptosis (13). Analysis was performed on Becton Dickinson FACSCanto instruments and data were analysed using FCS Express 5 Flow Cytometry RUO and Origin Pro 9.1G Software. Detailed flow cytometry methods have been published elsewhere (14, 15).

Proliferation assay

PBMC from patients with SS were labelled with CellTrace™ Far Red (Life Technologies; #C34564) and subsequently activated with T Cell Activation/Expansion Kit (Miltenyi Biotec #130–091–441) according to the manufacturer's instructions. The assay was performed in 96-well flat-bottom plates and a total volume of 200 μl. Proliferation was determined 24, 48 and 72 h after activation. Detailed proliferation methods are available elsewhere (16).

RESULTS

CD164 cell surface expression on total CD4⁺ T helper lymphocytes from patients with SS was significantly upregulated compared with healthy controls and patients with benign inflammatory skin disease, namely psoriasis (Fig. 1A). The medium fluorescence intensity (MFI) of CD164 on total lymphocytes from patients with SS was approximately 1200, while the MFI of CD164 on total lymphocytes from healthy donors was approximately 800. As expected, the expression of CD164 was not homogenous on the lymphocyte population of patients with SS. In most cases, CD4⁺CD26⁺ benign T helper

lymphocytes remained CD164⁺, while CD4⁺ lymphocytes with CD26 loss showed increased CD164 expression (Fig. 1B). In contrast, CD164 expression was, in most cases, independent of CD26 loss in control samples (Fig. 1C). In patients with SS, mean CD164 expression was 30.8% on average and varied between patients, ranging from 4.3% to 96.0% of CD4⁺ T cells. Mean CD164 expression in CD4⁺ T cells from healthy controls was 8.0% on average, ranging from 2.3% to 18.2%. Furthermore, CD164 expression on CD4⁺ T lymphocytes in blood from patients with SS was almost exclusively limited to CD4⁺CD26⁻ lymphocytes. The percentage of CD164⁺ T cells was not associated with disease duration, being the time of first diagnosis to either death (Pat. Nos 2–4, 7) or to discontinued observation at the time of manuscript preparation (Pat. Nos 1, 5–6, 8) (Fig. 1D).

As a next step, we analysed whether there was a difference in the level of expression of CD164 on malignant, clonal T cells compared with benign bystander T cells in the blood of individual patients with SS. The CD164 MFI on benign T cells in SS did not differ significantly from that on lymphocytes from healthy donors (MFI of 600 or 800, respectively). In contrast, CD164 expression was strongly upregulated (MFI of 1360) on the malignant T cells unequivocally identified by the expression of a specific Vβ clone (Fig. 2A). Moreover, in most cases (6 out of 8 patients), a high level of expression of CD164

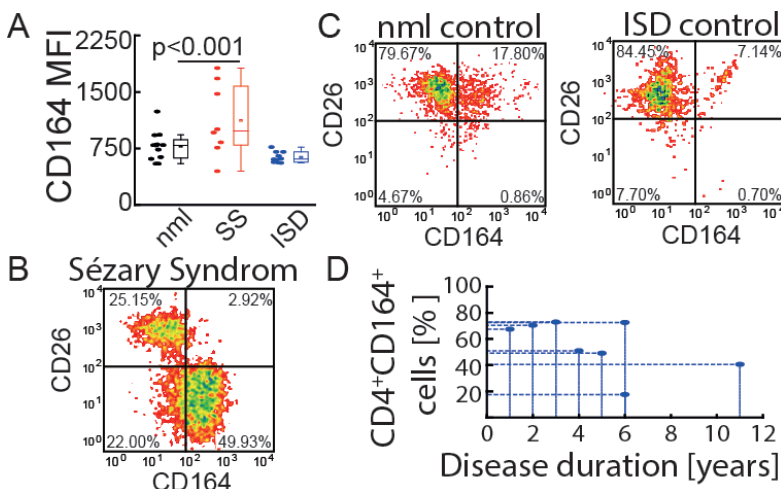


Fig. 1. CD164 expression is increased on CD4⁺ T cells from patients with Sézary syndrome (SS). (A) Flow cytometric analysis of CD164 expression in blood CD4⁺ T cells from healthy donors (nml), control population of patients with inflammatory skin disease (ISD; psoriasis) and from patients with SS. (B) Representative example of CD164 expression limited to CD4⁺CD26⁻ T cells in the blood of a patient with SS (plot gated on CD4). (C) Representative control sample gated on CD4⁺ cells from a healthy individual (nml) and a patient with inflammatory skin disease (ISD; psoriasis). (D) The percentage of CD164⁺ T cells in the blood of patients with SS do not correlate significantly with the duration of disease.

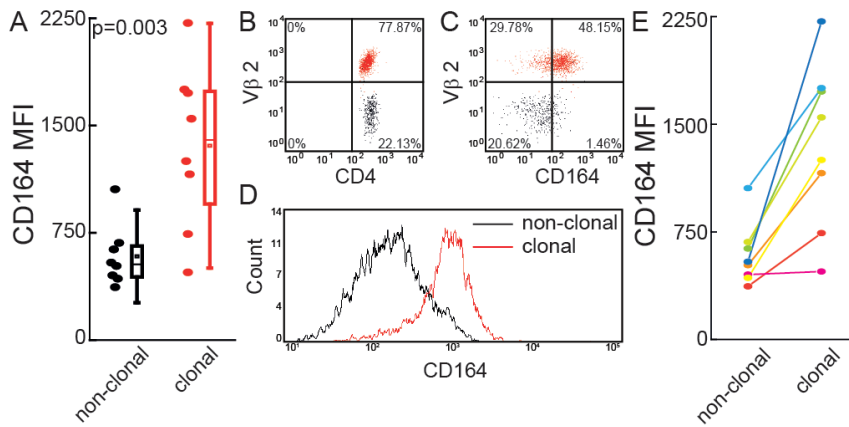


Fig. 2. Increased CD164 expression is restricted to malignant T cells from patients with Sézary syndrome (SS). (A) Clonal malignant T cells were definitively identified by staining with T-cell receptor V β antibodies specific for each patient's malignant clone. The CD164 expression of malignant (clonal) and benign (non-clonal) T cells was evaluated by flow cytometry analysis. (B) Representative example of flow cytometric identification of the malignant T-cell clone (red). (C) Representative example of increased CD164 expression limited to the malignant T-cell population (red). (D) Representative histograms demonstrate the increased mean fluorescent intensity of CD164 expression in clonal (red line) vs. non-clonal (black line) T cells from a patient with SS. (E) Pair-wise comparison of CD164 medium fluorescence intensity (MFI) in non-clonal and clonal CD4 $^{+}$ T cells in patients with SS. Each colour-indexed pair represents a data set from an individual patient with SS.

was limited to the malignant T cell clones, while the level of expression remained low on non-malignant cells (Fig. 2B–D). A more detailed pair-wise comparison of CD164 MFI in non-clonal vs. clonal CD4 $^{+}$ T cells in each individual patient with SS made it evident that in patient No. 3 CD164 expression on clonal malignant T cells (MFI=741.92) was not increased in comparison with healthy controls; however, it was still twice as high when intraindividually compared with non-clonal T cells from the same patient (MFI=370.37) (Fig. 2E; *dark orange* colour-indexed pair). In T cells from patient No. 4, a relatively low expression of CD164 and no intraindividual difference between non-clonal (MFI=452.9) and clonal (MFI=473.92) T cells could be detected.

In 3 representative patients with SS (Pat. Nos 1, 7, 8), further analysis revealed an increased apoptotic rate paralleled by reduced proliferation in CD164-expressing cells (Fig. 3).

DISCUSSION

The prognosis for SS is poor and curative treatment options, besides allogeneic stem cell transplan-

tation, are limited. Several new therapeutic options have attracted strong interest, as they may offer the possibility of developing new and effective targeted therapies. Open-label multicentre phase III clinical studies on anti-CD30 and anti-CCR4 in relapsed or refractory CTCL are active and recruiting and an open-label multicentre phase I study on a humanized anti-CD158k monoclonal antibody is in preparation (17–22). Nevertheless, other markers with potential for targeted approach in the treatment of CTCL are constantly being investigated.

CD164 is described as an adhesive sialomucin on CD34 $^{+}$ cells with the capacity to suppress haematopoietic cell proliferation (23). Here, we were able to show enhanced CD164 expression on malignant T cells in blood of patients with SS. In an

independent patients' sample, we found that CD164 expression on total lymphocytes from patients with SS was significantly upregulated compared with healthy controls. CD164 expression was in most cases limited to CD4 $^{+}$ CD26 $^{-}$ malignant T lymphocytes, unequivocally identified by the expression of a specific V β clone for each analysed patient.

These data are in line with the recent finding from Wysocka et al. (12) and suggest that increased expression of CD164 may be a promising diagnostic marker for SS. Based on our control population data, we propose that flow cytometric identification of >20% CD164 on CD4 $^{+}$ cells in the blood of erythrodermic patients should at least raise suspicion for SS.

Previous experimental studies have identified CD164 as a signalling receptor involved in proliferation, cell adhesion and migration in haematopoietic stem and progenitor cells (24, 25). Furthermore, recent functional experimental data have linked CD164 expression to progression of ovarian cancer (26), and document that targeting CD164 in D283-MED medulloblastoma cells and in colon cancer cell line HCT116 suppressed tumour proliferation, migration and invasion (27, 28).

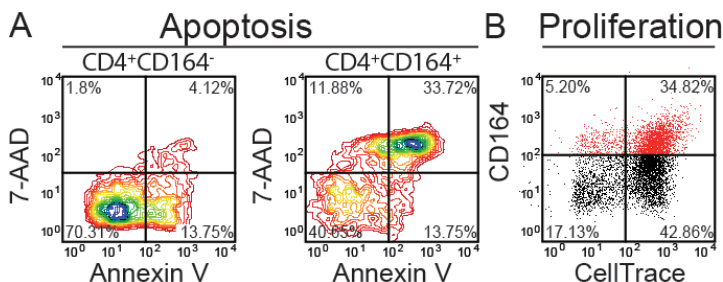


Fig. 3. Apoptosis and proliferation rate in CD164 expressing cells in patients with Sézary syndrome (SS). (A) Representative contour plots of increased annexin V $^{+}$ /7-AAD $^{+}$ late apoptotic CD164 $^{+}$ (right panel) vs. CD164 $^{-}$ (left panel) T cells from a patient with SS. (B) Representative dot plot of reduced proliferative activity in CD164 $^{+}$ (red dots) vs. CD164 $^{-}$ (black dots) T cells from a patient with SS. Twenty-four hours after induction of proliferation, proliferative activity can be detected in 14.93% [(5.2/34.82)*100%] of all CD164 $^{+}$ T cells and in 39.97% [(17.13/42.86)*100%] of all CD164 $^{-}$ T cells from a representative patient with SS.

This raises the possibility of a targeted CD164-linked therapeutic approach in SS.

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The authors declare no conflict of interest.

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